BAKER BOTTS LLP

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35.U.S.C. 371

EXPRESS MAIL LABEL No.	DATE
EF 377 399 171 US	January 10, 2002

ATTORNEY'S DOCKET NO. 34909-PCT-USA 069277.0108

U.S. APPLICATION NO. 10/031047

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/ESOO/00245	July 11, 2000	July 12, 1999

TITLE OF INVENTION: NUCLEIC ACIDS, VECTORS, AND CELL LINES COMPRISING A CYCLOOXYGENASE 2 (COX-2) PROMOTER AND METHODS OF

APPLICANT(S) FOR DO/EO/US: Manuel FRESNO ESCUDERO and Miguel Angel IÑIGUEZ PENA

Applicant herewith submits to the United States Designated /Elected Office (DO/EO/US) the following items and other information:

1. ☑ This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
3. ☑ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(I).
4. ☑ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☑ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☑ has been transmitted by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☑ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☑ A copy of the International Search Report (PCT/ISA/210)
   a. ☑ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☑ have been transmitted by the International Bureau
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.
8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [ ] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☑ A copy of the International Preliminary Examination Report (PCT/IPEA/409)
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☑ A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [ ] Other items or information:
    a. [ ] a copy of the International Search Report (PCT/ISA/210)
    b. [ ] a copy of the International Preliminary Examination Report (PCT/IPEA/409)
17. ☑ A substitute sequence listing.

10031047 .092302
10/031047
531 Rec'd PCT/PT 10 JAN 2002

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/ESOO/00245	July 11, 2000	July 12, 1999

17. [ ] The following fees are submitted:

Basic National Fee (37 CFR 1.492(a)(1)-(5):

Neither international preliminary examination fee (37 CFR 1.482)

Nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO (1.492(a)(3)) $1,040

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO (1.492(a)(5) $890.00

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO(1.492(a)(2)) $740.00

International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) (1.492(a)(1)) $710.00

International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) $ 100.00

CALCULATIONS PTO USE ONLY

ENTER APPROPRIATE BASIC FEE AMOUNT				=	$ 100
Surcharge of $130.00 for furnishing the oath or declaration later than **[ ] 20 [ ] 30** months from the earliest claimed priority date (37 C.F.R. 1.492(e)).					$ 130
Claims	**Number Filed**	**Number Extra**	**Rate**		$
Total Claims	16 -20=	0	X $ 18.00		$ 0
Independent Claims	6 -3=	3	X $ 84.00		$ 252
Multiple dependent claim(s) (if applicable)			+ $280.00		$
TOTAL OF ABOVE CALCULATIONS				=	$ 482
Reduction by ½ for filing by small entity, if applicable.					$
SUBTOTAL				=	$ 482
Processing fee of $130.00 for furnishing the English translation later than **[ ] 20 [ ] 30** months from the earliest claimed priority date (37 CFR 1.492(f)). +					$
TOTAL NATIONAL FEE				=	$ 482
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property +					$
TOTAL FEES ENCLOSED				=	$ 482
					Amt. refunded $
					charged $

a. [✓] A check in the amount of $ 482.00 to cover the above fees is enclosed.

b. [ ] Please charge our Deposit Account No. 02-4377 in amount of $_____ to cover the above fees. A copy of this sheet is enclosed.

c. [✓] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 02-4377. A copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:
Alicia A. Russo
BAKER BOTTS L.L.P.
30 Rockefeller Plaza
New York, New York 10112-4498

Alicia A. Russo

Attorney: Alicia A. Russo PTO Reg: 46,192

January 10, 2002
Date

BAKER BOTTS LLP

Attorney Docket Number: 34909-PCT-USA 069277.0108

Title: NUCLEIC ACIDS, VECTORS, AND CELL LINES COMPRISING A CYCLOOXYGENASE 2 (COX-2) PROMOTER AND METHODS OF SCREENING FOR COX-2 INHIBITORS

Use Space Below for Additional Information:

Amendments to the instant application have been made under Articles 19 and 30 including submission of a substitute specification in English. Applicants believe, therefore, that an English translation of the original PCT application in Spanish is not required.

AP34909-PCT-USA 069277.0108
PATENT

#5

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	FRESNO ESCUDERO et al.			
Serial No.	:	10/031,047	Examiner	:	TBA
Filed	:	January 10, 2002	Group Art Unit	:	TBA
For	:	NUCLEIC ACIDS, VECTORS, AND CELL LINES COMPRISING A CYCLOOXYGENASE 2 (COX-2) PROMOTER AND METHODS OF SCREENING FOR COX-2 INHIBITORS			

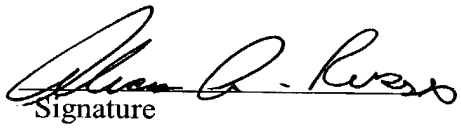
RESPONSE TO NOTICE OF MISSING REQUIREMENTS UNDER 35 U.S.C. §371

I hereby certify that this paper is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231

September 17, 2002
Date of Deposit

Alicia A. Russo
Attorney Name

46,192
PTO Registration No.


Signature

September 17, 2002
Date of Signature

Assistant Commissioner for Patents
Washinton, D.C. 20231

Dear Sir:

This paper is being filed in response to the Notice of Missing Requirements Under 35 U.S.C. §371 dated April 1, 2002. Applicants request a four-month extension of time and enclose the fee required under 37 C.F.R. §1.17(a)(4). Applicants enclose herewith a

corrected English translation of pages 28 and 29 and a Combined Declaration and Power of Attorney.

IN THE CLAIMS

Please **replace** pages 28 and 29 of the English translation as filled with the substitute pages 28 and 29 enclosed herewith.

Please **cancel** claims 1-9 listed on substitute pages 28 and 29.

Please **renumber** claims 9-24 added by the January 10, 2002 Preliminary Amendment as claims 10-25 as follows:

10. (AMENDED) A nucleic acid molecule having the sequence of from about nucleotide -1796 to about +104 of a human cyclooxygenase 2 gene operatively linked to a reporter gene.

11. (AMENDED) The nucleic acid molecule of claim 10, wherein the sequence is set forth by SEQ ID. NO:5.

12. (AMENDED) The nucleic acid molecule of claim 10, wherein the reporter gene is selected from the group consisting of a luciferase gene, a chloramphenicol acetyltransferase gene, and a β-galactosidase gene.

13. (AMENDED) The nucleic acid molecule of claim 10, wherein the nucleic acid molecule is contained in a vector.

14. (AMENDED) A nucleic acid molecule comprising about 1.9 kb of a human cyclooxygenase 2 promoter operatively linked to a reporter gene.

15. The nucleic acid molecule of claim 14, wherein the promoter has the sequence set forth by SEQ ID. NO:5.

16. (AMENDED) The nucleic acid molecule of claim 14, wherein the reporter gene is selected from the group consisting of a luciferase gene, a chloramphenicol acetyltransferase gene, and a β-galactosidase gene.

17. (AMENDED) The nucleic acid molecule of claim 14, wherein the nucleic acid molecule is contained in a vector.

18. (AMENDED) A cell comprising a nucleic acid molecule having the sequence of from about nucleotide -1796 to about +104 of a human cyclooxygenase 2 gene operatively linked to a reporter gene.

19. (AMENDED) The cell of claim 18, wherein the cell is a human cell.

20. (AMENDED) The cell of claim 19, wherein the cell is a Jurkat cell.

21. (AMENDED) The cell of claim 18, wherein the expression of the reporter gene is controlled by the sequence of the human cyclooxygenase 2 gene.

22. (AMENDED) The cell of claim 21, wherein the cell is capable of expressing the reporter gene.

23. (AMENDED) A cell line having the access number ECACC 9903245.

24. (AMENDED) An *Escherichia coli* DH5 cell line having the access number CECT 5145.

25. (AMENDED) A method comprising:

contacting a cell comprising a nucleic acid molecule comprising about 1.9 kb of a human cyclooxygenase 2 promoter operatively linked to a reporter gene with a test agent; and

measuring the reporter gene activity

wherein a reduction in reporter gene activity indicates that the test agent may be a transcriptional inhibitor of the human cyclooxygenase 2 gene.

REMARKS

This paper is being filed in response to the Notice of Missing Requirements Under 35 U.S.C. §371 dated April 1, 2002 (hereinafter "Notice"). Applicants request a four-month extension of time and enclose the fee required under 37 C.F.R. §1.17(a)(4). Applicants enclose herewith a corrected English translation of pages 28 and 29 and a Combined Declaration and Power of Attorney.

The Notice indicates that Applicants must provide an English translation having the same number of claims as the International Application; an oath or declaration; and a sequence listing in paper and computer readable form.

The International Application appears to have 9 claims while the English translation provided appears to have 8 claims. Applicants have enclosed herewith substitute pages 28 and 29 of the English translation having 9 claims. Applicant's Preliminary Amendment filed with the application papers on January 10, 2002 cancelled original claims 1-8 and added new claims numbered 9-24. The presence of a 9th claim in the substitute pages 28 and 29 results two claims numbered "9". Applicants have cancelled the "original" claim 9 recited on substitute page 29 and requested that the claims added by Applicant's January 10, 2002 Preliminary Amendment be renumbered 10-25 accordingly. Renumbered claims appear in the preceding "IN THE CLAIMS" section. Attached hereto is a marked-up version of the changes made by the instant amendment captioned "VERSION WITH MARKINGS TO SHOW CHANGES MADE" and is included pursuant to 37 C.F.R. §1.121(c)(ii).

Applicants filed a Sequence Listing in paper and computer readable form at the time the application was filed in the United States Patent and Trademark Office. The Notice did not identify any defects in these submissions. In a telephone call on July 8, 2002 to Guy F.

Birkenmeier, a Scientific Advisor to Applicant's Attorneys, Examiner Shakeel Ahmed stated that there were no defects in the Sequence Listing previously filed and, therefore, no Sequence Listing was required with this Response.

Applicants enclose the fee required for a four-month extension of time under 37 C.F.R. §1.17(a)(4) and providing a translation of the application later than 30 months from the priority date under 37 C.F.R. §1.492(f). Applicants do not believe any additional fee is required for this filing. However, any fees required for this submission not otherwise enclosed herewith may be charged to Deposit Account No. 02-4377. Two copies of this page are enclosed.

A copy of the Notice is enclosed herewith.

Respectfully submitted,

September 17, 2002

Louis S. Sorell
PTO Reg. No. 32,439

Alicia A. Russo
PTO Reg. No. 46,192

Attorneys for Applicants
BAKER BOTTS, L.L.P.
30 Rockefeller Plaza
New York, NY 10112
(212) 408-2626

Enclosures

VERSION WITH MARKINGS TO SHOW CHANGES MADE

This marked-up version was prepared with DeltaView software (v2.5.163). In this section, added text is marked with double underlining. *e.g.* added text, and deleted text is marked by a single strikethrough, *e.g.* ~~deleted text~~.

IN THE CLAIMS

Claims 9-24 presented in Applicant's Preliminary Amendment have been renumbered as follows:

10. ~~9.~~ (AMENDED) A nucleic acid molecule having the sequence of from about nucleotide -1796 to about +104 of a human cyclooxygenase 2 gene operatively linked to a reporter gene.

11. ~~10.~~ (AMENDED) The nucleic acid molecule of claim ~~9,~~10, wherein the sequence is set forth by SEQ ID. NO:5.

12. ~~11.~~ (AMENDED) The nucleic acid molecule of claim ~~9,~~10, wherein the reporter gene is selected from the group consisting of a luciferase gene, a chloramphenicol acetyltransferase gene, and a -galactosidase gene.

13. ~~12.~~ (AMENDED) The nucleic acid molecule of claim ~~9,~~10, wherein the nucleic acid molecule is contained in a vector.

14. ~~13.~~ (AMENDED) A nucleic acid molecule comprising about 1.9 kb of a human cyclooxygenase 2 promoter operatively linked to a reporter gene.

15. ~~14.~~ The nucleic acid molecule of claim ~~13,~~14, wherein the promoter has the sequence set forth by SEQ ID. NO:5.

16. ~~15.~~ (AMENDED) The nucleic acid molecule of claim ~~13,~~14, wherein the reporter gene is selected from the group consisting of a luciferase gene, a chloramphenicol acetyltransferase gene, and a - galactosidase gene.

17. ~~16.~~ (AMENDED) The nucleic acid molecule of claim ~~13,~~14, wherein the nucleic acid molecule is contained in a vector.

18. ~~17.~~ (AMENDED) A cell comprising a nucleic acid molecule having the sequence of from about nucleotide -1796 to about +104 of a human cyclooxygenase 2 gene operatively linked to a reporter gene.

19. ~~18.~~ (AMENDED) The cell of claim ~~17,~~18, wherein the cell is a human cell.

20. ~~19.~~ (AMENDED) The cell of claim ~~18,~~19, wherein the cell is a Jurkat cell.

21. ~~20.~~ (AMENDED) The cell of claim ~~17,~~18, wherein the expression of the reporter gene is controlled by the sequence of the human cyclooxygenase 2 gene.

22. ~~21.~~ (AMENDED) The cell of claim ~~20,~~21, wherein the cell is capable of expressing the reporter gene.

23. ~~22.~~ (AMENDED) A cell line having the access number ECACC 9903245.

24. ~~23.~~ (AMENDED) An *Escherichia coli* DH5 cell line having the access number CECT 5145.

25. ~~24.~~ (AMENDED) A method comprising:

contacting a cell comprising a nucleic acid molecule comprising about 1.9 kb of a human cyclooxygenase 2 promoter operatively linked to a reporter gene with a test agent; and

measuring the reporter gene activity

wherein a reduction in reporter gene activity indicates that the test agent may be a transcriptional inhibitor of the human cyclooxygenase 2 gene.

CLAIMS

1. A DNA construct that comprises all or part of a promoter sequence of the gene coding for cyclooxygenase 2 (cox-2) and a reporter gene, operatively joined to each other, such that said promoter sequence of the cox-2 gene controls the expression of said reporter gene in response to a suitable stimulus.

2. A construct according to claim 1, in which said promoter sequence of the cox-2 gene originates from the human cox-2 gene.

3. A construct according to claim 2, in which said promoter sequence of the cox-2 gene consists of a sequence lying between the nucleotide (-)1796 and the nucleotide (+)104 of the promoter of human cox-2.

4. A construct according to claim 1, in which said reporter gene is selected from the luciferase gene, the chloranphenicol acetyltransferase gene and the gene of beta galactosidase.

5. A vector that comprises a DNA construct according to any of claims 1 to 4.

6. A cell line that comprises a construct according to any of claims 1 to 4 or transformed with a vector according to claim 5.

7. A cell line according to claim 6, in which said cell line is derived from a cell line of human origin.

8. A cell line according to claim 7, in which said cell line of human origin is a line of Jurkat cells.

9. An assay method for the search for compounds that selectively inhibit the induction at a transcriptional level of cyclooxygenase-2 by a suitable stimulus, that comprises bringing a cell line according to any of claims 6 to 8, into contact with a compound whose potential selective inhibitory activity of induction at a transcriptional level of cox-2 it is wanted to assay, in conditions that allow the transcription of cox-2, and detecting, and/or measuring, the signal indicative of the expression of activity due to the reporter gene.

34909-PCT-USA 069277.0108
PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	FRESNO ESCUDERO et al.			
Serial No.	:	TBA	Examiner	:	TBA
Filed	:	July 11, 2000	Group Art Unit	:	TBA
For	:	CELL LINE COMPRISING THE PROMOTER OF CYCLOOXYGENASE-2 (COX-2) AND A WITNESS GENE, AND USE THEREOF IN THE SEARCH OF COX-2 TRANSCRIPTIONAL INDUCTION SELECTIVE INHIBITORS			

PRELIMINARY AMENDMENT AND SUBMISSION OF SEQUENCE LISTING

Hon. Commissioner of Patents and Trademarks
P.O. Box 2327
Arlington, VA 22202-3513

Box PCT

Dear Sir:

Applicants request entry of the following amendments prior to examination of the above-identified application on the merits.

IN THE TITLE

Please **amend** the title with the following rewritten title:

--NUCLEIC ACIDS, VECTORS, AND CELL LINES COMPRISING A CYCLOOXYGENASE 2 (COX-2) PROMOTER AND METHODS OF SCREENING FOR COX-2 INHIBITORS--

IN THE SPECIFICATION

Please **insert** the following paragraph at page 1after the title of the invention:

--This application is a national stage application under 35 U.S.C. §371 of international application PCT/ES00/00245 filed July 11, 2000 and published as WO 01/04350 in Spanish which claims priority to Spanish application ES P9901557 filed July 12, 1999.--

Please **amend** the paragraph beginning at page 7, line 3 and ending at page 7, line 6 with following rewritten paragraph:

--Figure 1 shows the sequence of the promoter zone of the cox-2 gene (SEQ ID. NO:3). The arrows indicate the sequences of hybridization of the oligonucleotides used in the PCR (polymerase chain reaction).--

Please **amend** the paragraph beginning at page 7, line 8 and ending at page 7, line 10 with following rewritten paragraph:

--Figure 2 shows the strategy for cloning the promoter region of the cox-2 gene in the pXP2 plasmid in order to obtain the construct prom2-1906-LUC. The oligonucleotide depicted corresponds to SEQ ID. NO:4.--

Please **delete** the Sequence Listing at pages 31-32 and substitute therefor, the Substitute Sequence Listing included herewith in paper and computer form.

IN THE CLAIMS

Please **delete** Claims 1-8.

Please **add** the following new claims.

9. (NEW) A nucleic acid molecule having the sequence of from about nucleotide –1796 to about +104 of a human cyclooxygenase 2 gene operatively linked to a reporter gene.

10. (NEW) The nucleic acid molecule of claim 9, wherein the sequence is set forth by SEQ ID. NO:5.

11. (NEW) The nucleic acid molecule of claim 9, wherein the reporter gene is selected from the group consisting of a luciferase gene, a chloramphenicol acetyltransferase gene, and a β-galactosidase gene.

12. (NEW) The nucleic acid molecule of claim 9, wherein the nucleic acid molecule is contained in a vector.

13. (NEW) A nucleic acid molecule comprising about 1.9 kb of a human cyclooxygenase 2 promoter operatively linked to a reporter gene.

14. The nucleic acid molecule of claim 13, wherein the promoter has the sequence set forth by SEQ ID. NO:5.

15. (NEW) The nucleic acid molecule of claim 13, wherein the reporter gene is selected from the group consisting of a luciferase gene, a chloramphenicol acetyltransferase gene, and a β-galactosidase gene.

16. (NEW) The nucleic acid molecule of claim 13, wherein the nucleic acid molecule is contained in a vector.

17. (NEW) A cell comprising a nucleic acid molecule having the sequence of from about nucleotide –1796 to about +104 of a human cyclooxygenase 2 gene operatively linked to a reporter gene.

18. (NEW) The cell of claim 17, wherein the cell is a human cell.

19. (NEW) The cell of claim 18, wherein the cell is a Jurkat cell.

20. (NEW) The cell of claim 17, wherein the expression of the reporter gene is controlled by the sequence of the human cyclooxygenase 2 gene.

21. (NEW) The cell of claim 20, wherein the cell is capable of expressing the reporter gene.

22. (NEW) A cell line having the access number ECACC 9903245.

23. (NEW) An *Escherichia coli* DH5 cell line having the access number CECT 5145.

24. (NEW) A method comprising:

contacting a cell comprising a nucleic acid molecule comprising about 1.9 kb of a human cyclooxygenase 2 promoter operatively linked to a reporter gene with a test agent; and

measuring the reporter gene activity

wherein a reduction in reporter gene activity indicates that the test agent may be a transcriptional inhibitor of the human cyclooxygenase 2 gene.

REMARKS

Applicant requests entry of the instant amendment prior to examination of the above-identified patent application on the merits.

Claims 9-23 are pending in the above-identified application. Claims 1-8 have been cancelled. New claims 9-23 have been added by the instant amendment.

Attached hereto is a marked-up version of the changes made by the instant amendment. The attached page is captioned "VERSION WITH MARKINGS TO SHOW CHANGES MADE" and is only included for the Examiner's convenience. Should any discrepancies be discovered, the version presented in the preceding "IN THE SPECIFICATION" section shall be deemed to be correct.

Applicants assert that these new claims are fully supported by the application as filed and do not constitute new matter.

Applicants submit herewith a Substitute Sequence Listing in paper and computer form. I hereby state that the content of the paper and computer readable copies of the Substitute Sequence Listing submitted in accordance with 37 C.F.R. §1.821(c) and (e), are the same. I hereby state that the content of the paper and computer readable copies of the Substitute Sequence Listing, submitted in accordance with 37 C.F.R. §1.821(g), herein does not include new matter.

Amendments to the instant application have been made previously under Patent Cooperation Treaty Articles 19 and 30 including submission of a substitute specification in English. Applicants believe, therefore, that an English translation of the original PCT application in Spanish is not required.

34909-PCT-USA 069277.0108
PATENT

The Commissioner is hereby authorized to charge any fees due with this submission not otherwise enclosed herewith to Deposit Account No. 02-4377. Please credit any overpayment of fees associated with this filing to the above-identified deposit account. A duplicate of this page is enclosed.

Respectfully submitted,

Louis Sorell
PTO Reg. No. 32,439

Alicia A. Russo
PTO Reg. No. 46,192

Attorneys for Applicants
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30 Rockefeller Plaza
New York, NY 10112
(212) 408-2500

34909-PCT-USA 069277.0108
PATENT

The Commissioner is hereby authorized to charge any fees due with this submission not otherwise enclosed herewith to Deposit Account No. 02-4377. Please credit any overpayment of fees associated with this filing to the above-identified deposit account. A duplicate of this page is enclosed.

Respectfully submitted,

Alicia A. Russo

Louis Sorell
PTO Reg. No. 32,439

Alicia A. Russo
PTO Reg. No. 46,192

Attorneys for Applicants
BAKER BOTTS, L.L.P.
30 Rockefeller Plaza
New York, NY 10112
(212) 408-2500

VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the following sections, added text is marked with double underlining. e.g. added text, and deleted text is marked by a single strikethrough, e.g. ~~deleted text~~.

IN THE TITLE

Please **amend** the title with the following rewritten title:

-- NUCLEIC ACIDS, VECTORS, AND CELL ~~LINE~~ LINES COMPRISING ~~THE PROMOTER OF~~ A CYCLOOXYGENASE-2 (COX-2) PROMOTER ~~AND A WITNESS GENE, AND USE THEREOF IN THE SEARCH OF COX-2 TRANSCRIPTIONAL INDUCTION SELECTIVE~~ AND METHODS OF SCREENING FOR COX-2 INHIBITORS

IN THE SPECIFICATION

The paragraph beginning at page 7, line 3 and ending at page 7, line 6 has been amended as follows:

--Figure 1 shows the sequence of the promoter zone of the cox-2 gene (SEQ ID. NO:3). The arrows indicate the sequences of ~~hybridisation~~ hybridization of the oligonucleotides used in the PCR ~~reaction [polymerase chain reaction]~~ (polymerase chain reaction).--

The paragraph beginning at page 7, line 8 and ending at page 7, line 10 has been amended as follows:

--Figure 2 shows the strategy for cloning the ~~promoting~~ promoter region of the cox-2 gene in the pXP2 plasmid in order to obtain the construct prom2-1906-LUC. The oligonucleotide depicted corresponds to SEQ ID. NO:4.--

<110> FRESNO ESCUDERO, Manuel
IÑIGUEZ PENA, Miguel Angel

<120> NUCLEIC ACIDS, VECTORS, AND CELL LINES COMPRISING A CYCLOOXYGENASE 2 (COX-2) PROMOTER AND METHODS OF SCREENING FOR COX-2 INHIBITORS

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CELL LINE THAT COMPRISES THE PROMOTER OF CYCLOOXYGENASE-2 (COX-2) AND A REPORTER GENE, AND USE THEREOF IN THE SEARCH FOR SELECTIVE INHIBITORS OF THE TRANSCRIPTIONAL INDUCTION OF COX-2

FIELD OF THE INVENTION

This invention relates, in general, to the search for products with potential therapeutic applications. In particular, the invention relates to a method for the search for compounds that selectively inhibit the induction at a transcriptional level of cyclooxygenase-2 that comprises the use of a cell line that expresses in a stable manner a construct of DNA in which the gene promoter sequence of cyclooxygenase-2 controls the expression of a reporter gene in response to appropriate stimuli.

BACKGROUND OF THE INVENTION

The cyclooxygenase (cox) is an enzyme implicated in numerous processes. Two isoforms of cox are known, cyclooxygenase 1 (cox-1) and cyclooxygenase 2 (cox-2). Although both isoforms are related to the production of prostaglandins implicated in physiological processes, it seems that cox-2 is the isoform predominately implicated in various pathologies such as inflammation, carcinogenesis, angiogenesis and certain neurodegenerative processes.

Induction at a transcriptional level of cox-2 occurs

in response to several factors, among which can be found the expression of oncogenes, the treatment of tumours with promoters, mytogenous, pro-inflammatory stimuli, growth factors and cytokines [reviewed by Smith and DeWitt, 1996; Griswold and Adams, 1996; Jouzeau et al., 1997 (see section relating to REFERENCES)]. In most cases, the induction of this enzyme translates into an increase in the synthesis of prostaglandins, although other modes of action cannot be discarded.

The capacity of certain drugs of the family of non-steroidal anti-inflammatory drugs (NSAIDs) to inhibit cox-2 explains their therapeutic effects [reviewed by Smith and DeWitt, 1996; Griswold and Adams, 1996; Jouzeau et al., 1997]. Similarly, there is growing evidence that the inhibition of cox-2 both by NSAIDS and by glucocorticoids or by cyclosporin A has immunosuppressive effects [Iñiguez et al., 1998; Hall and Wolf, 1997; Zhou et al., 1994; and the reviews cited earlier]. Other actions of induction of cox-2 relate to the implication of this enzyme in cancer, angiogenesis and neurodegenerative processes such as Alzheimer's disease. It has been found that both inhibition of the induction at a transcription level of cox-2 and the enzymatic inhibition of cox-2 attenuate these processes [Shiff et al., 1996; Tsujii et al., 1997 y 1998; Subbaramiah et al., 1998; Pasinetti, 1998].

After discovering the inducible cox-2 isoform of the cyclooygenase enzyme, the methods for identification of new anti-inflammatory drugs have focussed on selecting

compounds that are selective inhibitors of the enzymatic activity of cox-2 against the constitutive isoform cox-1. There are several types of system for this. Some use *in vitro* assays with purified or semi-purified cox-2 [Famaey, 1997; Noreen et al., 1998]. Other authors use animal or human cell lines that predominantly express the cox-2 isoforms in natural conditions or after induction with stimuli [Famaey, 1997; Berg et al., 1997]. Some use cell lines of animal or human origin in which the cox-2 protein is over-expressed by means of stable transfection of cDNA coding for this protein [Lora et al., 1997; O´Neill et al., 1995; Cromlish and Kennedy, 1996]. In some cases, it has been possible to determine using mRNA analysis whether such compounds inhibit the induction of cox-2 at a transcriptional level [Tao et al., 1998; Subbaramiah et al., 1998]. Systems have also been established for studying inhibitory compounds by means of *in vivo* assays, either with whole blood or with purified cells from healthy donors [Famaey, 1997; Brideau et al., 1996].

In any case, the main limitation of these systems lies in the fact that they allow selection of compounds that inhibit the enzymatic activity of the cox-2 enzyme, without considering their effects on the induction of the production of the protein, the step prior to production of prostaglandins by this enzyme. In addition to this limitation, it has been shown that the relative potencies of these compounds vary for the same drug for different types of assay. Similarly, an important aspect for consideration concerns the inhibition of the

physiological activity of cox-2, which would also be inhibited by the type of compounds identified by the aforementioned systems, which could lead to adverse side effects.

It is known from **WO 98/37235** a method of screening agents as candidates for drugs which suppress induction of COX-2 promoter. This screening procedure relies on the use of a recombinant vector comprising a construct containg 1432 bases from the COX-2 transcription start site (-1432 to +59) ligated to the luciferase gene.

Document **Huang, J-C and M.Y. Dawood (1988) Fertility and Sterility 70:734-739** discloses the determination of the effect of PMA and IL-1β on COX-2 promoter activity based on the measurement of luciferase activity generated in endometirial stromal cells transfected with a vector that contains a luciferase reporter and a 900-base pair promoter sequence (-891 to +9 relative to the transcription site) corresponding to the human gene.

Document **Inoue, H. and T. Tanabe (1998) Biochem. Biophys: Res. Comm. 244: 143-148** studies the transcriptional role of the NF-kB site of the COX-2 gene in U937 cells employing luciferase reporter vector driven by the human COX-2 promoter region (nucleotides -327 to +59) stably transfected into U937 cells.

There is therefore a need to develop a method for searching for compounds that selectively inhibit induction of cox-2 at a transcriptional level by different stimuli that overcomes the drawbacks mentioned above.

OVERVIEW OF THE INVENTION

This invention provides a solution to the existing need that consists of developing an assay system for the search of compounds that selectively inhibit the induction of cox-2 at a transcriptional level by different stimuli. This criterion allows compounds to be selected that inhibit the production of cox-2, and so will act as inhibitors of the actions derived from an increase in the expression of cox-2 and of the subsequent increase in the production of prostaglandins that set off various pathological processes. Among other processes, the following processes can be highlighted: inflammatory processes, uncontrolled cellular proliferation, angiogenesis, carcinogenesis and neurodegenerative pathologies, as were described earlier. The criterion for the selection of compounds according to this invention lies in the inhibition of the inducible activity of the promoter of cox-2. Therefore, those compounds that inhibit the physiological basal activity of production of cox-2 will not be selected.

For the development of the solution provided by this invention, it has been necessary to construct a cell line that expresses in a stable fashion a construct of DNA in which the promoter sequence of the cox-2 gene controls

the expression of the reporter gene in response to a suitable stimulus. The regulation of the expression of the cox-2 gene is determined by the regulatory activity of its promoter, while the measurement of the activity of the reporter gene provides an indirect measure of the activity of the promoter of cox-2 in response to different agents.

Thus, an object of this invention constitutes a DNA construct (or recombinant DNA) that comprises a promoting sequence of the cox-2 gene and a reporter gene, operatively joined to each other, such that said promoter sequence of the gene controls the expression of the reporter gene in response to a suitable stimulus.

An additional object of this invention constitutes a vector, such as a plasmid or an expression vector, that comprises said DNA construct.

Another additional object of this invention constitutes a cell line that contains said DNA construct, or said plasmid that contains said DNA construct, that expresses it in a stable fashion.

Finally, another additional object of this invention constitutes a method for the search for compounds that selectively inhibit the induction of cyclooxygenase-2 at a transcriptional level, that comprises the use of said cell line that expresses said DNA construct in a stable fashion.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the sequence of the promoter zone of the cox-2 gene. The arrows indicate the sequences of hybridisation of the oligonucleotides used in the PCR reaction [polymerase chain reaction].

Figure 2 shows the strategy for cloning the promoting region of the cox-2 gene in the pXP2 plasmid in order to obtain the construct prom2-1906-LUC.

Figure 3 shows the results of the analysis by RT-PCR [reverse transcription-polymerase chain reaction] of the expression of mRNA of cox-2 in Jurkat cells. In Figure 3(A) the effect of treatment with PMA and PMA + calcium ionophore A23187, hereinafter PMA-Ion, on the expression of mRNA of cox-2 is shown. In Figure 3(B) the inhibition by cyclosporin of the transcriptional induction of cox-2 is shown. In both cases, the result obtained for the non-inducible mRNAs of the cox-1 isoform and the glycerol-aldehyde dehydrogenase (GAPDH) by way of control is shown.

Figure 4 shows the result of the stimulation by PMA or by PMA+Ion of the luciferase activity in Jurkat cells transitorily transfected with the prom2-1906-LUC construct. As a control, it is checked that both the prom1-898-LUC construct and the empty plasmid PXP2 are not inducible.

Figure 5 shows the result of inhibition by

cyclosporin A (CsA) of the stimulation caused by PMA-Ion of the construct prom2-1906-LUC in the transient transfection in Jurkat cells.

Figure 6 shows the results of an experiment of transitory transfection with the prom2-1906-LUC construct and treatment with dexamethasone.

Figure 7 shows the results of the luciferase activity of different clones obtained after stable transfection with the prom2-1906-LUC construct.

Figure 8 shows the results of inhibition by cyclosporin A (CsA) of the stimulation by PMA+Ion of the luciferase activity of the stable clones of Jurkat-1906LUC.

Figure 9 shows the results obtained for the inhibition by the glucocorticoide dexamethasone (Dex) on the stimulation by PMA+Ion of the luciferase activity of the stable clones of Jurkat-1906LUC.

Figure 10 shows the inhibition by Resveratrol (Res) of the induction of luciferase activity of the stable clones as a control of the assay system.

DETAILED DESCRIPTION OF THE INVENTION

This invention provides a DNA construct (or recombinant DNA) that comprises the whole part of a promoter sequence of the cyclooxygenase 2 (cox-2) gene

and a reporter gene, operatively joined to each other, such that said promoter sequence of the cox-2 gene controls the expression of said reporter gene in response to a suitable stimulus.

The promoter sequence of the cox-2 gene may be of any origin, although preferably said sequence will proceed from the human cox-2 gene.

As a reporter gene, any reporter gene may be used that is able to produce an easily detectable signal, chosen from among those normally used in these types of transfection assays, for example, the chloranphenicol acetyl transferase (CAT) gene, the beta galactosidase (β-gal) gene, the luciferase gene, for example, from glow-worm or from *Renilla*. In a particular embodiment of this invention, said reporter gene is the luciferase gene of glow-worm because of its extreme sensitivity, speed and ease of use and low cost of the assay for its detection.

The invention also provides a vector, such as a plasmid or an expression vector, which contains the aforementioned DNA construct. In principle, any vector can be used that is suitable for inserting into said DNA construct. These vectors are useful for the transformation of cells.

The cell line provided by this invention comprises, and expresses in a stable fashion, said DNA construct that comprises all or part of a promoter sequence of the cox-2 gene and a reporter gene, operatively joined to

each other, such that said promoter sequence of the cox-2 gene controls the expression of said reporter gene in response to a suitable stimulus.

The transformed cell line that contains the aforementioned DNA construct may originate from any suitable cell line able to express said DNA construct in a stable fashion, for example, a cell line of human origin such as a line of cells of the T-lymphocyte type, Hep-G2 cells derived from hepatocellular carcinoma, Hela cells derived from an adenocarcinoma of the cervix, cells of monocyte-macrophage type, for example, the lines U937 and THP-1, etc. In a particular embodiment of this invention, a Jurkat cell line has been selected (originally described by Schneider et al., 1977) as a representative example of a transformed cell line of the T-lymphocyte type as a model for studying the expression of a gene related to the immune response. In addition, said cell line is easy to grow and provides a high yield of cells per unit time and volume (ml) of culture.

The cell line provided by this invention can be used as an assay system in the search and identification (screening) of compounds that selectively inhibit the induction at a transcriptional level of cox-2 by different stimuli.

The cell line provided by this invention can be easily obtained using conventional procedures of Genetic Engineering, for example, by means of a process that comprises (i) the isolation of a promoter sequence of the

cox-2 gene, (ii) the cloning of said sequence in a vector that contains the reporter gene, in a position in which said promoter sequence is able to control the expression of said reporter gene, and (iii) the transfection of a suitable cell line with said plasmid. In Example 3, there is described in detail a specific way for obtaining individual clones of transformed Jurkat cells which express the reporter gene (luciferase) in a stable fashion, denominated Jurkat-C3-1906LUC, Jurkat-F9-1906LUC and Jurkat-C7-1906LUC, in which the basal luciferase activity was determined and it was checked that the expression of the reporter gene (luciferase) was being induced in response to the same stimuli as the promoter of cox-2 as had been previously established with transitorily transfected cells.

The assay system (cell line) provided by this invention has been previously validated by the transitory transfection of the prom2-1906-LUC construct and the analysis of the luciferase activity under different stimuli and inhibitors. The results obtained were compared with a non-inducible control of a similar construct in which, instead of the promoter of cox-2, the promoter of the cox-1 isoform was put in place and with the empty vector pXP2. Under the same conditions, the behaviour of the endogenous cox-2 gene was also determined by experiments of RT-PCR in which the expression of mRNA is analysed. As a non-inducible control, the behaviour of the endogenous gene of the cox-1 isoform and the non-inducible gene of glycerol-aldehyde-phosphate dehydrogenase (GAPDH) was determined.

The main treatments were activators such as phorbol ester PMA (10 ng/ml) and the combination of PMA and calcium ionophore A23187 [PMA+Ion] (1 μM). Treatment with drugs that inhibit the induction by PMA+Ion were carried out with dexamethasone (1 μM), and with cyclosporin A (100 ng/ml).

Example 2 includes some validation assays of the assay system provided by this invention in transitory transfection, as well as the expression of the endogenous genes in Jurkat cells. Also, in Examples 3 and 4, some assays are presented carried out with the stable clones obtained, with compounds that induce and inhibit the induction the cox-2 promoter.

The assay system provided by this invention is useful for the search and identification (screening) of compounds that selectively inhibit the induction at a transcriptional level of cox-2 by different stimuli. This type of selective inhibitor of cox-2 may have numerous potential therapeutic applications as the implications derived from the induction of cox-2 not only affect the inflammatory response, but also processes related to uncontrolled cellular proliferation and formation of tumours (for example, the appearance of adenomas, cancer of the colon and the development of polyps and angiogenesis, among others), with immunosuppressant actions and with neurodegenerative processes such as Alzheimer's disease. As a result, it might be supposed that the compounds that selectively inhibit the transcriptional induction of cox-2 may be

useful as anti-inflammatory agents, as compounds able to attenuate uncontrolled cellular proliferation and/or the process of tumorigenesis, as immunosuppressants or as potential drugs with therapeutic properties in Alzheimer's disease.

The invention also provides an assay method for searching and identifying (screening) compounds that selectively inhibit the induction at a transcriptional level of cox-2 by a suitable stimulus (described in Example 4) which comprises bringing the cell line provided by this invention (assay system) into contact with the compound to be assayed, in other words, with the compound whose potential selective inhibitory activity of the induction at a transcriptional level of cox-2 it is wanted to test, in conditions that allow the transcription of cox-2, and detecting, and/or measuring, the signal indicative of the expression of activity due to the reporter gene. Alternatively, if so desired, the assay method object of this invention can be performed by bringing the cell line, the assay system and the compound that activates transcription induction of cox-2 into contact.

In the assay method provided by this invention, the regulation of the expression of the cox-2 gene is determined by the regulatory activity of its promoter, while the activity of the reporter gene provides an indirect measure of the activity of the cox-2 promoter in response to different agents.

The assay method for the search and identification of compounds that selectively inhibit the induction at a transcriptional level of cox-2 by an appropriate stimulus provided by this invention allows the selection of compounds that inhibit the production of cox-2 by means of a criterion based on the inhibition of the inducible activity of the cox-2 promoter, which will not select those compounds that inhibit the basal physiological activity of the production of cox-2.

The following examples serve to illustrate preferred embodiments of the invention, but they should not be considered as limiting the scope thereof.

EXAMPLE 1

Production of a DNA construct that comprises a promoter sequence of cox-2 and the luciferase gene

1.1 Cloning the promoter of cox-2

In the first place, the promoter sequence of the human cox-2 gene was cloned from the sequence described by [Tazawa et al., 1994] represented in Figure 1.

The technique of polymerase chain reaction (PCR) was used, with the initiating oligonucleatides or "primers" designed for selective amplification of the fragment of DNA corresponding to the promoter sequence of this gene. The template DNA used was genomic DNA from the Jurkat human lymphocyte cell line. The oligonucleotides used were those identified as SEC.ID.No.: 1 and SEC.ID.No.: 2 [see the section concerning the LIST OF SEQUENCES].

These oligonucleotides amplify a sequence that ranges from nucleotide -1796 to nucleotide +104 of the promoter zone of the cox-2 gene (see Figure 1). For the PCR reaction, the *Advantage cDNA PCR kit* [Clontech] was used with 30 cycles repeated every 45 seconds at 94°C and 3 minutes at 68° C in a PTC-200 thermocycler PTC-200 [MJ Research].

1.2 Construction of the expression vector

The fragments generated after amplification were subcloned into the plasmid pXP2 [Nordeen, 1988] which contains the sequence that codes for the luciferase gene which will be used as the reporter gene (see Figure 2). The oligonucleotides were designed such that at the 5' end they contain an additional recognition sequence for restriction enzymes. After amplification, double chain ends are generated that contain the restriction targets BamHI at the 5' end and Bg1II at the 3' end. The pXP2 vector contains a Bg1II target at the multiple cloning site, which generates ends compatible both with Bg1II and BamHI ends. After digestion of the insert obtained by PCR containing the promoting sequence with the Bg1II and BamHI enzymes and the pXp2 vector with Bg1II, the binding of these sequences was performed. In this fashion, the plasmid prom2-1906-LUC was obtained in which the sequence (-)1796-(+)104 of the cox-2 promoter is located in front of the luciferase gene, controlling its expression. This construction was sequenced to check the fidelity of the promoter sequence and to verify the cloning site.

Example 2

Experiments in analysis of the regulation of the activity of the promoter of cox-2 by means of experiments with RT-PCR and transitory transfection of the prom2-1906-LUC construct in the Jurkat cell line

In order to study the regulation of the expression of the endogenous cox-2 gene in the Jurkat cell line, the analysis of the expression of its mRNA was performed by experiments with RT-PCR. At the same time, in order to validate the prom2-1906-LUC construct and to check that the regulation of the cloned promoter is as expected, experiments were carried out of transitory transfection of the prom2-1906-LUC construct using the Jurkat cell line. In both experiments, the cells were treated with compounds that stimulate and inhibit the induction of the promoter of cox-2.

Treatment with activator compounds were carried out using the phorbol ester PMA (Phorbol 12-Myristate 13-Acetate) (10 ng/ml) (Sigma) and the combination of PMA and Calcium ionophore A23187 (1μM) (Sigma), hereinafter PMA+Ion.

Treatment with drugs that inhibit the induction by PMA+Ion were carried out with cyclosporin A (CsA) (100 ng/ml) or the synthetic glucocorticoide Dexamethasone (Dex) (1μM) (Sigma).

2.1 <u>Regulation of the expression of cox-2 mRNA in Jurkat cells</u>

The results obtained from the analysis by RT-PCR of the expression of mRNA of cox-2 in Jurkat cells are presented in Figure 3, where the following can be observed:

a) treatment with PMA (10 ng/ml) produces a slight increase in the expression of the mRNA of cox-2, while the combination treatment with PMA+Ion leads to a greater increase in the expression of this gene at a transcriptional level [Figure 3(A)]; and

b) inhibition by CsA (100 ng/ml) of the transcriptional induction of cox-2 [Figure 3(B)].

As a control in both cases, the result obtained for the non-inducible mRNAs of the cox-1 isoform and for the glycerol aldehyde phosphate dehydrogenase (GAPDH) are shown.

2.2 Evaluation of the luciferase activity under different stimuli

The luciferase activity in Jurkat cells transitorily transfected with the prom2-1906-LUC construction using the Lipofectin agent [Life Technologies] has been analysed. The cells were stimulated with PMA (10 ng/m) (Sigma) and with the combination PMA+Ion (1 μM) (Sigma).

As a non-inducible control, a similar construct was used in which, instead of the cox-2 promoter, the promoter of the cox-1 isoform was put in place [prom1-898-LUC] and with the empty vector pXP2.

The luciferase activity was determined using the kit

"Luciferase Assay System" [Promega], with $1x10^6$ cells which where lysed in 50 µl of lysis buffer. In the extracts obtained, the light emission produced was measured using a luminometer Monolight 2010 [Analytical Luminiscence Laboratory] with an automatic injection system of 100 µl of reagent.

The results obtained are shown in Figure 4. As can be seen in said figure, the results obtained in this assay are comparable to those obtained in the analysis of mRNA of cox-2 [Example 2.1], in other words, treatment with PMA+Ion produces a greater increase in the number of times there is induction of the luciferase activity (approximately 12 times the basal value). These data show that the cloned promoter sequence behaves in a similar fashion to the endogenous gene. As a control, it is checked that neither the prom1-898-LUC construct nor the empty plasmid pXP2 are inducible.

2.3 Evaluation of the luciferase activity under different inhibitors

The inhibition of stimulation by PMA (10 ng/m) (Sigma) or PMA+Ion (1 µM) (Sigma) on the luciferase activity in Jurkat cells transitorily transfected with the prom2-1906-LUC construct has been investigated by using the immunosuppressant drug cyclosporin A (CsA) (100 ng/ml). The results obtained are shown in Figure 5 where it can been seen that treatment with CsA (100 ng/ml) reduces stimulation of the promoter of cox-2 in response to PMA+Ion to values similar to basal ones.

Similarly, the inhibition of stimulation by PMA+Ion (1 μM) (Sigma) on the luciferase activity has been analysed in Jurkat cells transitorily transfected with the prom2-1906 construct by means of the glucocorticoide dexamethasone (1 μM) (Sigma). The results obtained are shown in Figure 6 where it can be seen that treatment with dexamethasone (1 μM) (Dex) reduces stimulation of the promoter of cox-2 in response to PMA+Ion.

Example 3

Production of a cell line that stably expresses a DNA construct that comprises a promoter sequence of cox-2 and the luciferase gene.

For the creation of a cell line that stably expresses the prom2-1906-LUC construct, Jurkat cells were co-transfected with the vector prom2-1906-LUC and a vector denominated pcDNA3.1/Hygro (Invitrogen) which contains the gene for resistance to hygromycin. The transfection was carried out by means of the technique of electroporation in cuvettes of 0.4 cm (BioRad) with 15 x 10^6 cells in 0.5 ml of complete medium [RMPI medium supplemented with 10% of foetal serum, L-glutamine 2mM and a mixture of antibiotics] (All these products were acquired from Life Technologies). The cells were incubated over ice for 10 minutes with 25 μg of plasmid prom2-1906-LUC and 5 μg of the vector pCDNA3.1./Hygro. After this period, the cells were electroporated in a Gene Pulser II (BioRad) apparatus at 1.500 μFaradays of capacitance and a current of 280 V. Next, the cells were incubated over ice for 10 minutes before adding 10 ml of

complete medium. The cells were cultured in this medium in 75 cm^2 culture flasks (Nunc) for 48 hours in a cell incubator at 37° C with a humidity of 95% and 5% of CO_2. At this time, the medium was changed for complete medium with no antibiotics to which hygromycin (Boehringer Mannheim) was added at a concentration of 200 µg/ml. The cells were cultured in this medium for 30 days with successive changes of medium. During this period the resistant population that survived treatment with the selective antibiotic was selected, in other words, the cells stably transfected with the gene for resistance to the hygromycin antibiotic. In this population the expression of the luciferase gene was analysed in order to determine the presence of transfectants stable for the plasmid prom2-1906-LUC. For this, $1x10^6$ cells were lysed in 50 µl of lysis buffer (Promega) and with the extracts obtained, the luciferase activity was determined using the reagents contained in the kit of the "Luciferase Assay System" [Promega]. Measurement of the light produced was determined using a luminometer MonoLight 2010 (Analytical Luminiscence Laboratory) with a system of automatic injection of 100 µl of reagent.

From this polyclonal population (Jurkat-pool-1906LUC) a limit dilution was performed on 96-well plates in complete medium with hygromycin in order to obtain individual clones that expressed the luciferase gene in a stable fashion. These clones were grown until obtaining at least $1x10^6$ cells with which measurement of the luciferase activity could be performed as described earlier. In this way, three individual clones were

obtained denominated Jurkat-C3-1906LUC, Jurkat-F9-1906LUC and Jurkat-C7-1906LUC. In these clones the basal luciferase activity was determined in RLU (relative luminescence units) and it was checked that the expression of the luciferase reporter was being induced in response to the same stimuli as the promoter of cox-2 as had been established previously with cells transitorily transfected (see Figure 7). In the three clones, the basal values of luciferase activity increase from 3 to 6 times with a treatment of 6 hours with the phorbol ester PMA and up to 10 - 20 times with a combined treatment PMA+Ion, in a similar fashion to the results obtained in the cells transfected transitorily.

Example 4

Establishment of an assay system for compounds that regulate the expression of the gene cox-2 in the clones of the cell line that stably expresses a DNA construct that comprises a promoter sequence of cox-2 and the luciferase gene.

The clones were cultured on plates of 96-wells at a density of $.1x10^5$ cells in 200 µl of RPMI medium supplemented with 2% of foetal serum, L-Glutamine 2 mM and a mixture of antibiotics. The cells were treated for 6 hours with different concentrations of compounds whose activity it was hoped to analyse. In the case of the assay of the activity of these compounds on the induction of activity of the cox-2 promoter, the cells were treated with PMA+Ion for 5 hours, after 1 hour of pre-treatment with the compound to test. After this period, the cells

were lysed in 50 µl of lysis buffer and their luciferase activity determined using 20 µl in a luminometer as was described in Example 2.2. Next, some results obtained are shown with compounds previously described as inhibitors of the stimulation of the cox-2 promoter.

Figure 8 shows the results obtained with the compound cyclosporin A (CsA) which produces an inhibition of the stimulation obtained with PMA+Ion in stable Jurkat clones, in a similar fashion to that already described (Iñiguez et al., 1998), and that observed in the transitory transfections illustrated in Example 2.3.

Figure 9 shows the results obtained with the compound Dexamethasone (Dex), which, like glucocorticoide and anti-inflammatories, produces an inhibition of the stimulation obtained with PMA+Ion in the stable Jurkat clones, corresponding to that already described (Smith and DeWitt, 1996) and that observed previously in the transitory transfections of Example 2.3.

Figure 10 shows the results obtained with the compound Resveratrol (Res), recently described as an inhibitor of the stimulation by PMA of the gene cox-2 (Subbaramiah, et al., 1998). This compound produces an inhibition of the stimulation obtained with PMA+Ion om the stable Jurkat clones.

The validity of the assay system is thus demonstrated due to its similar behaviour for the clones obtained, for the transitory transfections and the

results obtained with endogenous mRNA. With the use of compounds whose activity on the cox-2 promoter is known, whether they be stimulatory or inhibitory, it is established that it is possible to detect both positively and negatively the basal or induced expression of the cox-2 gene with the assay system developed in present invention.

DEPOSIT OF BIOLOGICAL MATERIAL

A sample of a Jurkat cell line, denominated J-1906-F9, that stably expresses a DNA construct that comprises a promoter sequence of the cox-2 gene and the luciferase gene, has been deposited in the European Collection of Animal Cell Cultures (ECACC) [Salisbury, United Kingdom] on the 24 March 1999 and has been assigned the access number ECACC 99032405.

A sample of the plasmid prom2-1906-LUC, inserted into *Escherichia coli* DH5, denominated DH5 prom2-1906-LUC, has been deposited in the Spanish Collection of Culture Types (CECT) [Burjassot, Valencia] on the 24 March 1999 and has been assigned the access number CECT 5145.

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CLAIMS

1. A DNA construct that comprises the sequence lying between the nucleotide (-) 1796 and the nucleotide (+)104 of the promoter of human cyclooxygenase 2 (cox-2) gene and a reporter gene, operatively joined to each other, such that said promoter sequence of the cox-2 gene controls the expression of said reporter gene in response to a suitable stimulus.

2. A construct according to claim 1, in which said reporter gene is selected from the luciferase gene, the chloramphenicol acetyltransferase gene and the gene of beta galactosidase.

3. A vector that comprises a DNA construct according to any of claims 1 to 2.

4. A cell line that comprises a construct according to any of claims 1 to 2 or transformed with a vector according to claim 3.

5. A cell line according to claim 4, in which said cell line is derived from a cell line of human origin.

6. A cell line according to claim 5, in which said cell line of human origin is a line of Jurkat cells.

7. A cell line according to claims 4 to 6 which expresses in stable fashion the DNA construct of claims 1 to 2.

8. An assay method for the search for compounds that selectively inhibit the induction at a transcriptional level of cyclooxygenase-2 by a suitable stimulus, that comprises bringing a cell line according to any of claims 5 to 7, into contact with a compound whose potential selective inhibitory activity of induction at a transcriptional level of cox-2 it is wanted to assay, in conditions that allow the transcription of cox-2, and detecting, and/or measuring, the signal indicative of the expression of activity due to the reporter gene.

CELL LINE THAT COMPRISES THE PROMOTER OF CYCLOOXYGENASE-2 (COX-2) AND A REPORTER GENE, AND USE THEREOF IN THE SEARCH FOR SELECTIVE INHIBITORS OF THE TRANSCRIPTIONAL INDUCTION OF COX-2

ABSTRACT

The cell lines comprises a DNA construct that comprises all or part of a promoter sequence of the gene coding for cyclooxygenase 2 (cox-2) and a reporter gene, operatively joined to each other, such that said promoter sequence of the cox-2 gene controls the expression of said reporter gene in response to a suitable stimulus. The assay method comprises bringing said cell line into contact with the compound to be assayed and determining the existence of a signal indicative of the expression of activity due to the reporter gene. This method is suitable for the induction at a transcriptional level of cox-2 by suitable stimuli.

[SEC.ID.No.]: 1:

(i) CHARACTERISTICS OF THE SEQUENCE:

(A) LENGTH: 33 nucleotides

(B) TYPE: nucleic acid

(C) NO OF CHAINS: single chain

(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: DNA

(xi) DESCRIPTION OF THE SEQUENCE: SEC. ID. No.: 1:

GGGGGATCCG GATTCTAACA TGGCTTCTAA CCC 33

(2) INFORMATION ON THE IDENTIFIED SEQUENCE No.: 2:

(i) CHARACTERISTICS OF THE SEQUENCE:

(A) LENGTH: 30 nucleotides

(B) TYPE: nucleic acid

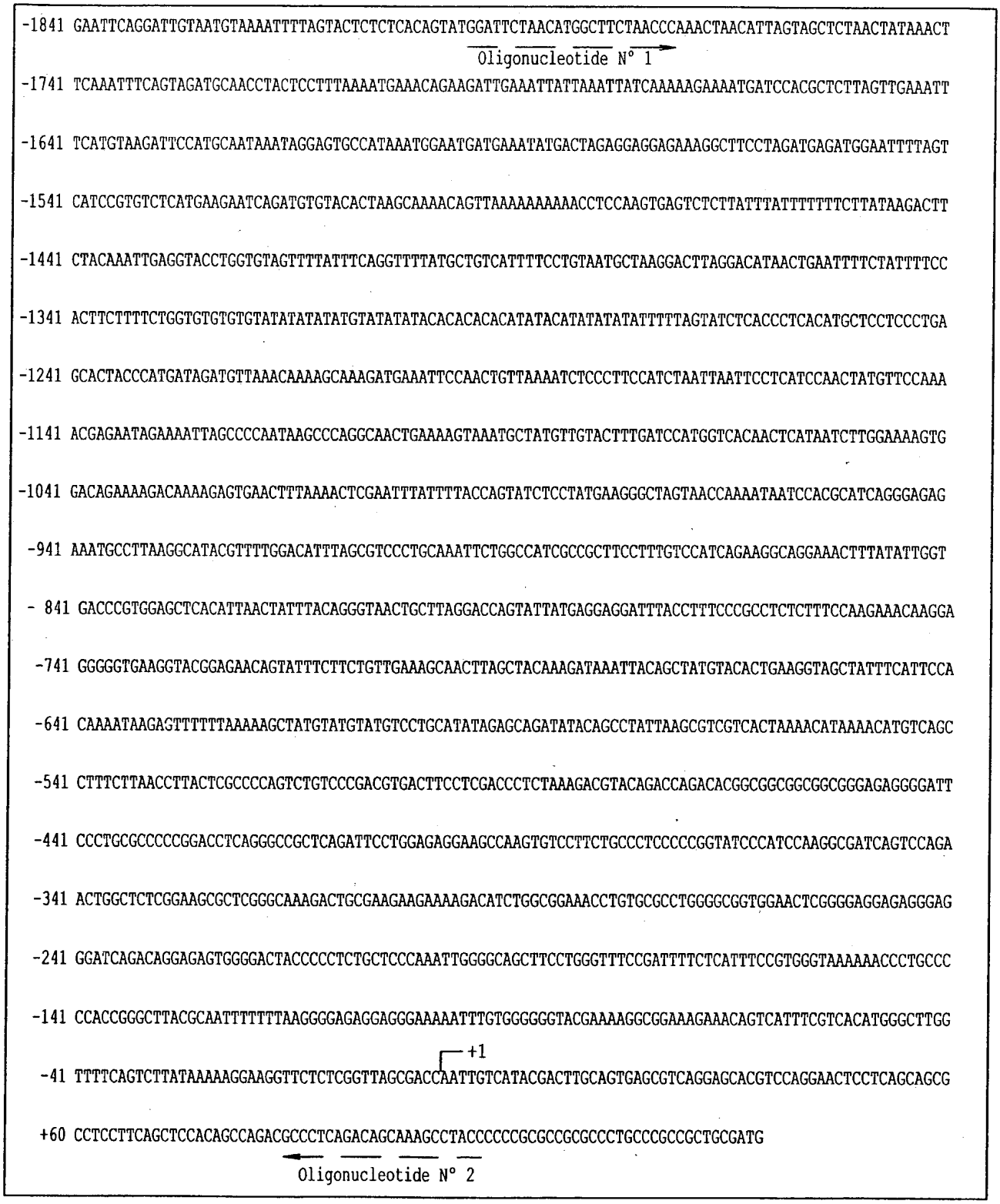
(C) NO OF CHAINS: single chain

(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: DNA

(xi) DESCRIPTION OF THE SEQUENCE: SEC. ID. No.: 2:

GGGAGATCTG GTAGGCTTTG CTGTCTGAGG 30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Oligonucleotide N° 1
-1741 TCAAATTTCAGTAGATGCAACCTACTCCTTTAAAATGAAACAGAAGATTGAAATTATTAAATTATCAAAAAGAAAATGATCCACGCTCTTAGTTGAAATT
-1641 TCATGTAAGATTCCATGCAATAAATAGGAGTGCCATAAATGGAATGATGAAATATGACTAGAGGAGGAGAAAGGCTTCCTAGATGAGATGGAATTTTAGT
-1541 CATCCGTGTCTCATGAAGAATCAGATGTGTACACTAAGCAAAACAGTTAAAAAAAAAACCTCCAAGTGAGTCTCTTATTTATTTTTTTCTTATAAGACTT
-1441 CTACAAATTGAGGTACCTGGTGTAGTTTTATTTCAGGTTTTATGCTGTCATTTTCCTGTAATGCTAAGGACTTAGGACATAACTGAATTTTCTATTTTCC
-1341 ACTTCTTTTCTGGTGTGTGTGTATATATATATGTATATATACACACACACATATACATATATATTTTTAGTATCTCACCCTCACATGCTCCTCCCTGA
-1241 GCACTACCCATGATAGATGTTAAACAAAAGCAAAGATGAAATTCCAACTGTTAAAATCTCCCTTCCATCTAATTAATTCCTCATCCAACTATGTTCCAAA
-1141 ACGAGAATAGAAAATTAGCCCCAATAAGCCCAGGCAACTGAAAAGTAAATGCTATGTTGTACTTTGATCCATGGTCACAACTCATAATCTTGGAAAAGTG
-1041 GACAGAAAAGACAAAAGAGTGAACTTTAAAACTCGAATTTATTTTACCAGTATCTCCTATGAAGGGCTAGTAACCAAAATAATCCACGCATCAGGGAGAG
-941 AAATGCCTTAAGGCATACGTTTTGGACATTTAGCGTCCCTGCAAATTCTGGCCATCGCCGCTTCCTTTGTCCATCAGAAGGCAGGAAACTTTATATTGGT
- 841 GACCCGTGGAGCTCACATTAACTATTTACAGGGTAACTGCTTAGGACCAGTATTATGAGGAGGATTTACCTTTCCCGCCTCTCTTTCCAAGAAACAAGGA
-741 GGGGGTGAAGGTACGGAGAACAGTATTTCTTCTGTTGAAAGCAACTTAGCTACAAAGATAAATTACAGCTATGTACACTGAAGGTAGCTATTTCATTCCA
-641 CAAAATAAGAGTTTTTTAAAAAGCTATGTATGTATGTCCTGCATATAGAGCAGATATACAGCCTATTAAGCGTCGTCACTAAAACATAAAACATGTCAGC
-541 CTTTCTTAACCTTACTCGCCCCAGTCTGTCCCGACGTGACTTCCTCGACCCTCTAAAGACGTACAGACCAGACACGGCGGCGGCGGCGGGAGAGGGGATT
-441 CCCTGCGCCCCCGGACCTCAGGGCCGCTCAGATTCCTGGAGAGGAAGCCAAGTGTCCTTCTGCCCTCCCCCGGTATCCCATCCAAGGCGATCAGTCCAGA
-341 ACTGGCTCTCGGAAGCGCTCGGGCAAAGACTGCGAAGAAGAAAAGACATCTGGCGGAAACCTGTGCGCCTGGGGCGGTGGAACTCGGGGAGGAGAGGGAG
-241 GGATCAGACAGGAGAGTGGGGACTACCCCCTCTGCTCCCAAATTGGGGCAGCTTCCTGGGTTTCCGATTTTCTCATTTCCGTGGGTAAAAAACCCTGCCC
-141 CCACCGGGCTTACGCAATTTTTTTAAGGGGAGAGGAGGGAAAAATTTGTGGGGGGTACGAAAAGGCGGAAAGAAACAGTCATTTCGTCACATGGGCTTGG
+1
-41 TTTTCAGTCTTATAAAAAGGAAGGTTCTCTCGGTTAGCGACCAATTGTCATACGACTTGCAGTGAGCGTCAGGAGCACGTCCAGGAACTCCTCAGCAGCG
+60 CCTCCTTCAGCTCCACAGCCAGACGCCCTCAGACAGCAAAGCCTACCCCCCGCGCCGCGCCCTGCCCGCCGCTGCGATG
Oligonucleotide N° 2

FIG. 1

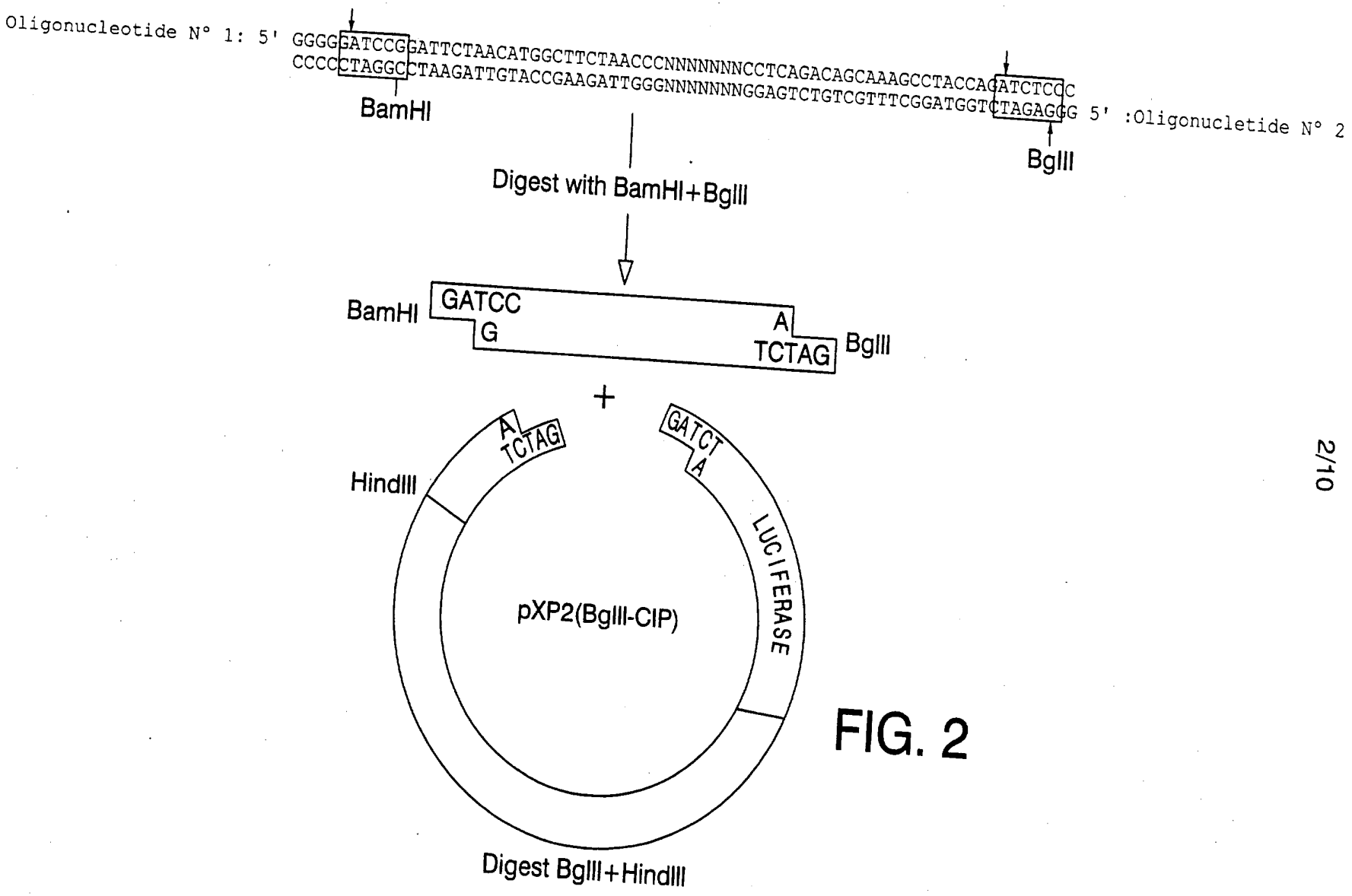

FIG. 2

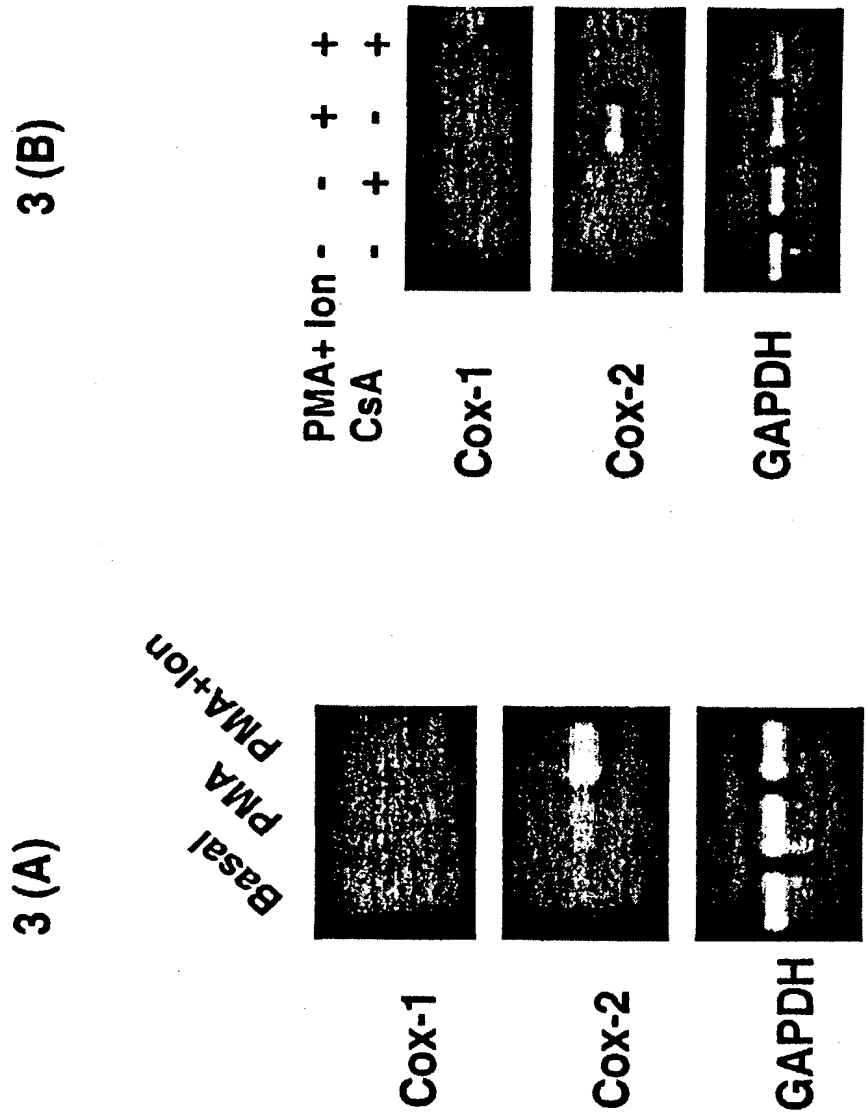
3 (A)
Basal
PMA
PMA+Ion
Cox-1
Cox-2
GAPDH
3 (B)
PMA+ Ion - - + + +
CsA - + - + +
Cox-1
Cox-2
GAPDH

FIG. 3


BASAL
PMA
PMA+ION
INDUCTION TIMES
0
2
4
6
8
10
12
14
pXP2
prom1-898LUC
prom2-1906LUC

FIG. 4

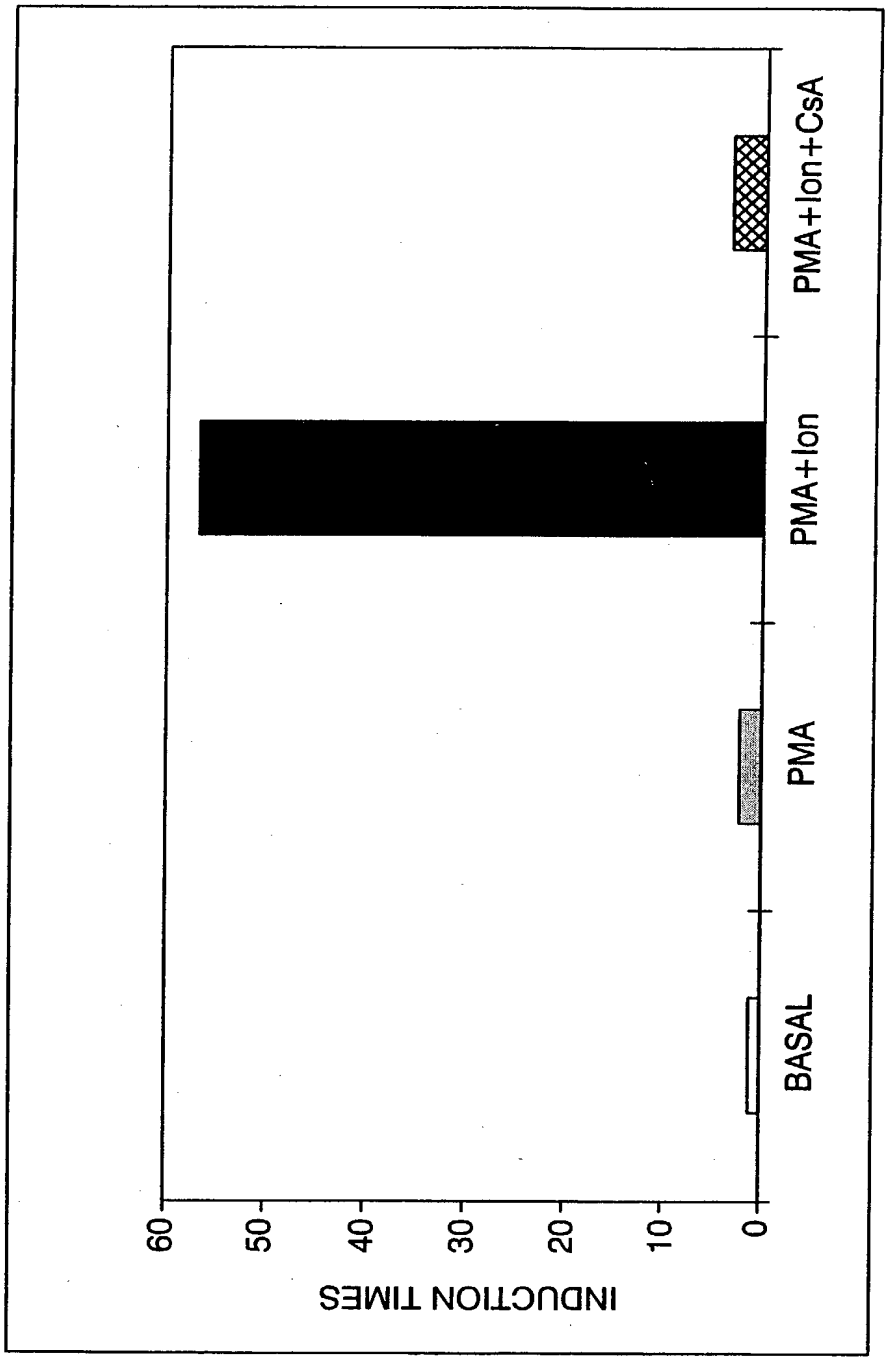
INDUCTION TIMES
0
10
20
30
40
50
60
BASAL
PMA
PMA+Ion
PMA+Ion+CsA

FIG. 5

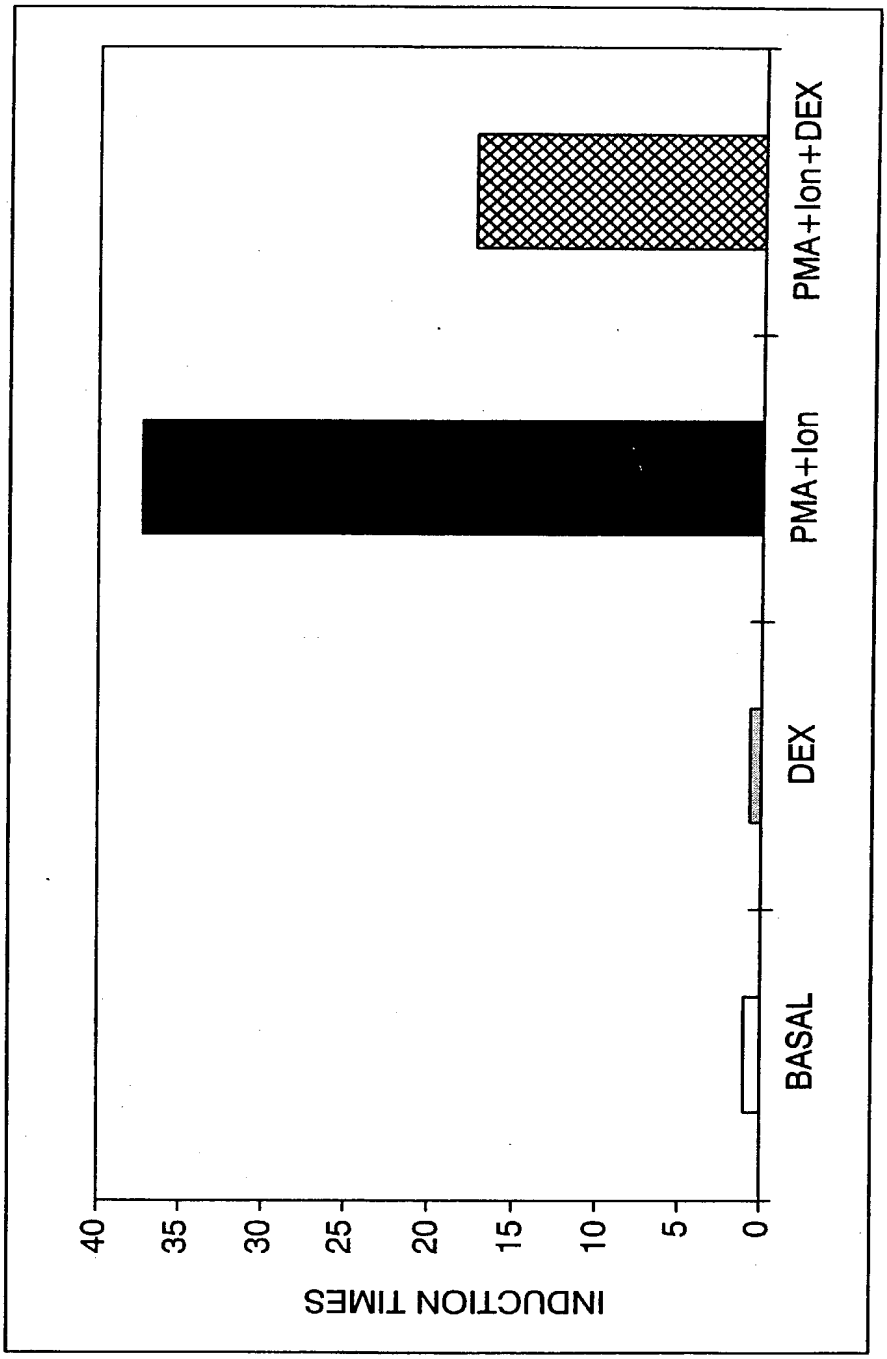
INDUCTION TIMES
0
5
10
15
20
25
30
35
40
BASAL
DEX
PMA+Ion
PMA+Ion+DEX

FIG. 6

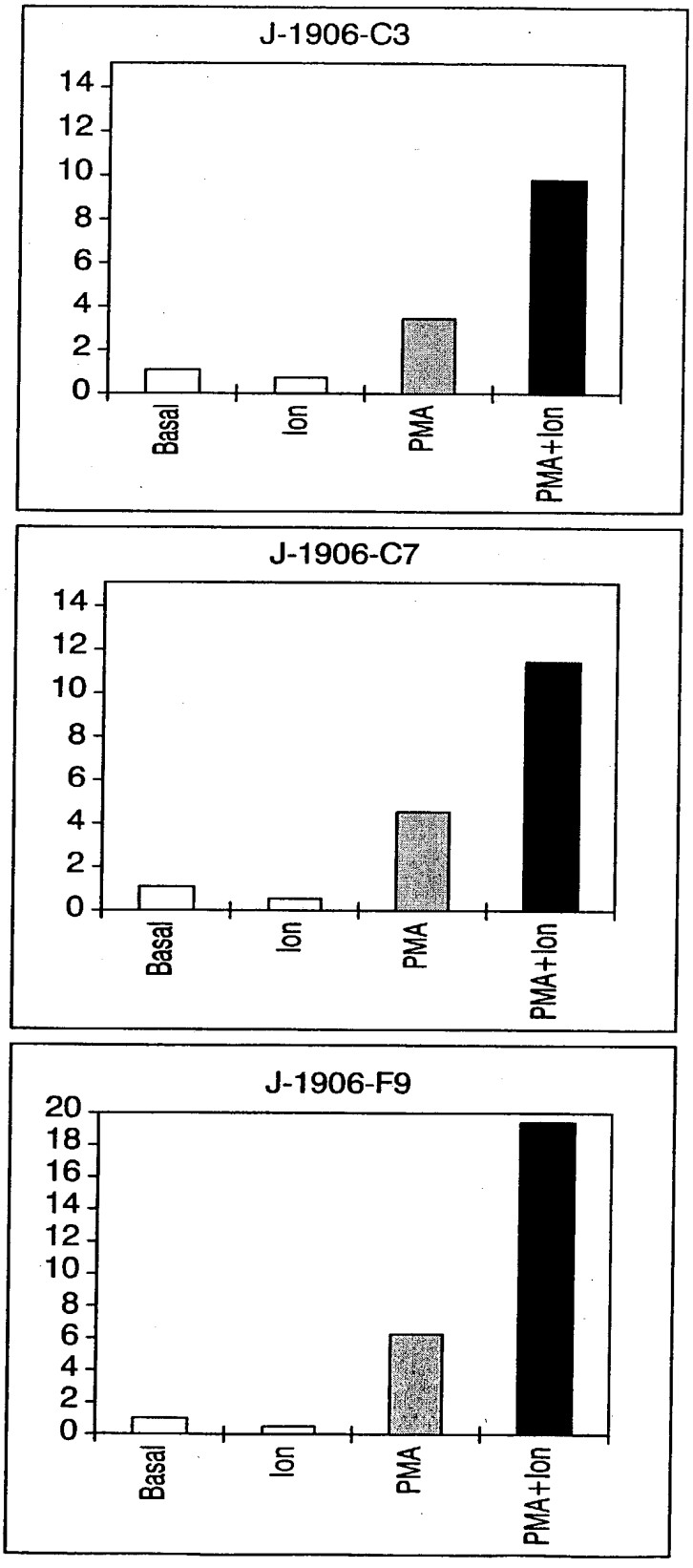
J-1906-C3
14
12
10
8
6
4
2
0
Basal
Ion
PMA
PMA+Ion
J-1906-C7
14
12
10
8
6
4
2
0
Basal
Ion
PMA
PMA+Ion
J-1906-F9
20
18
16
14
12
10
8
6
4
2
0
Basal
Ion
PMA
PMA+Ion

FIG. 7

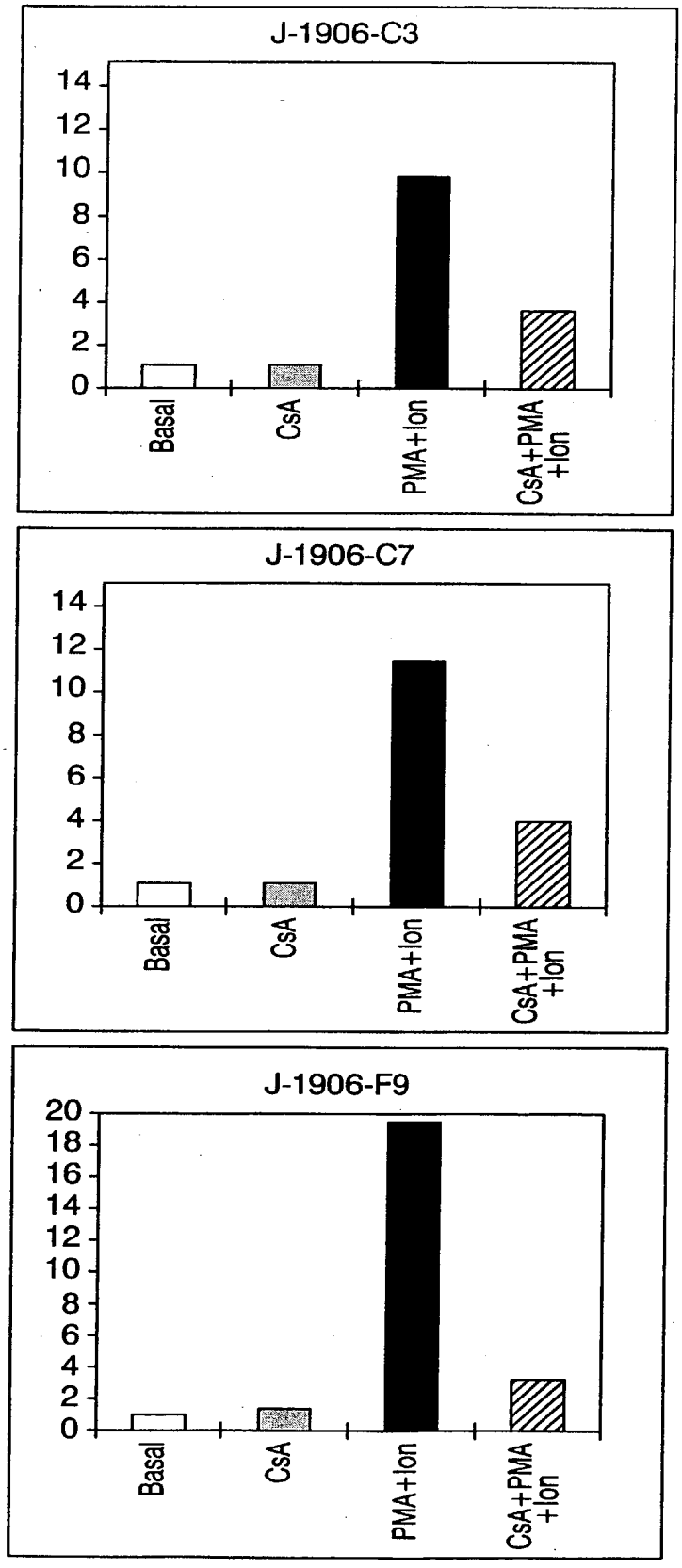
J-1906-C3
14
12
10
8
6
4
2
0
Basal
CsA
PMA+Ion
CsA+PMA +Ion
J-1906-C7
14
12
10
8
6
4
2
0
Basal
CsA
PMA+Ion
CsA+PMA +Ion
J-1906-F9
20
18
16
14
12
10
8
6
4
2
0
Basal
CsA
PMA+Ion
CsA+PMA +Ion

FIG. 8

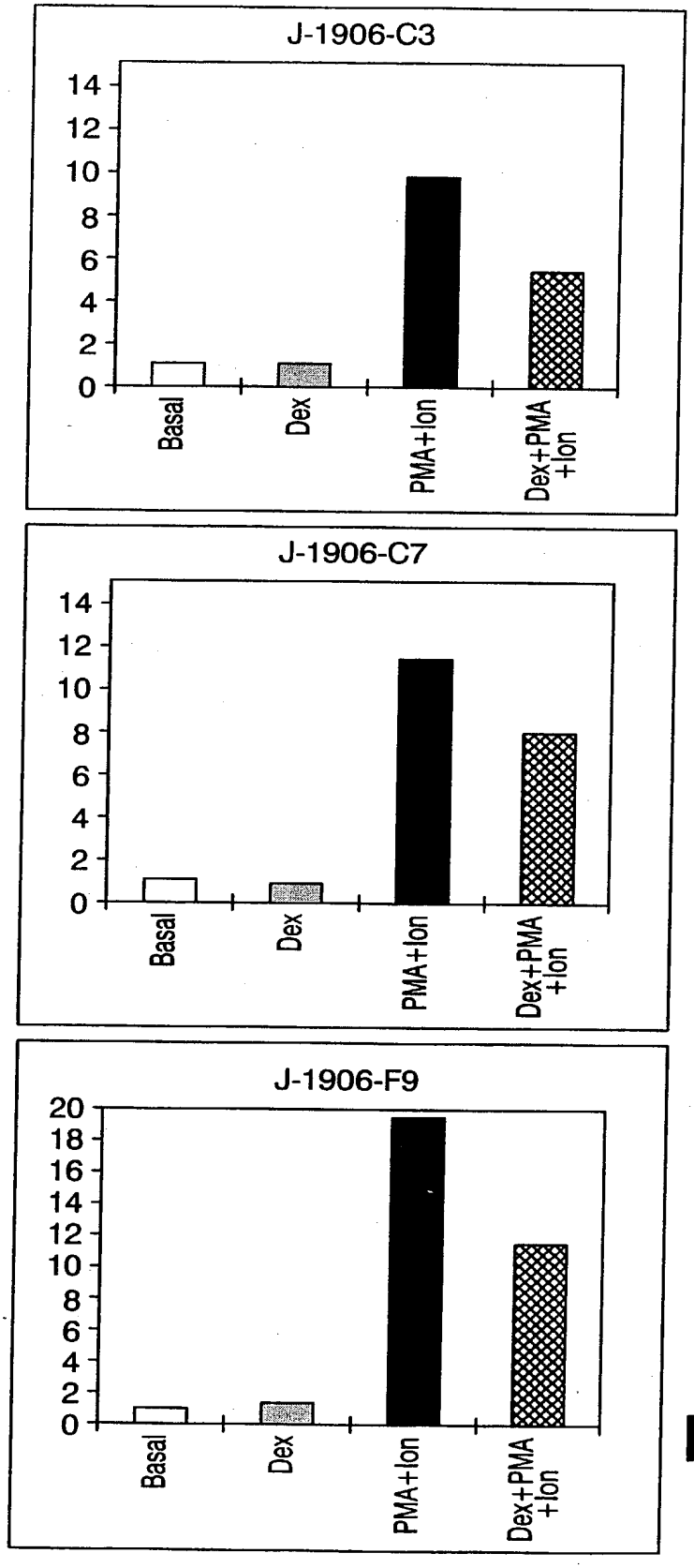
J-1906-C3
14
12
10
8
6
4
2
0
Basal
Dex
PMA+Ion
Dex+PMA +Ion
J-1906-C7
14
12
10
8
6
4
2
0
Basal
Dex
PMA+Ion
Dex+PMA +Ion
J-1906-F9
20
18
16
14
12
10
8
6
4
2
0
Basal
Dex
PMA+Ion
Dex+PMA +Ion

FIG. 9

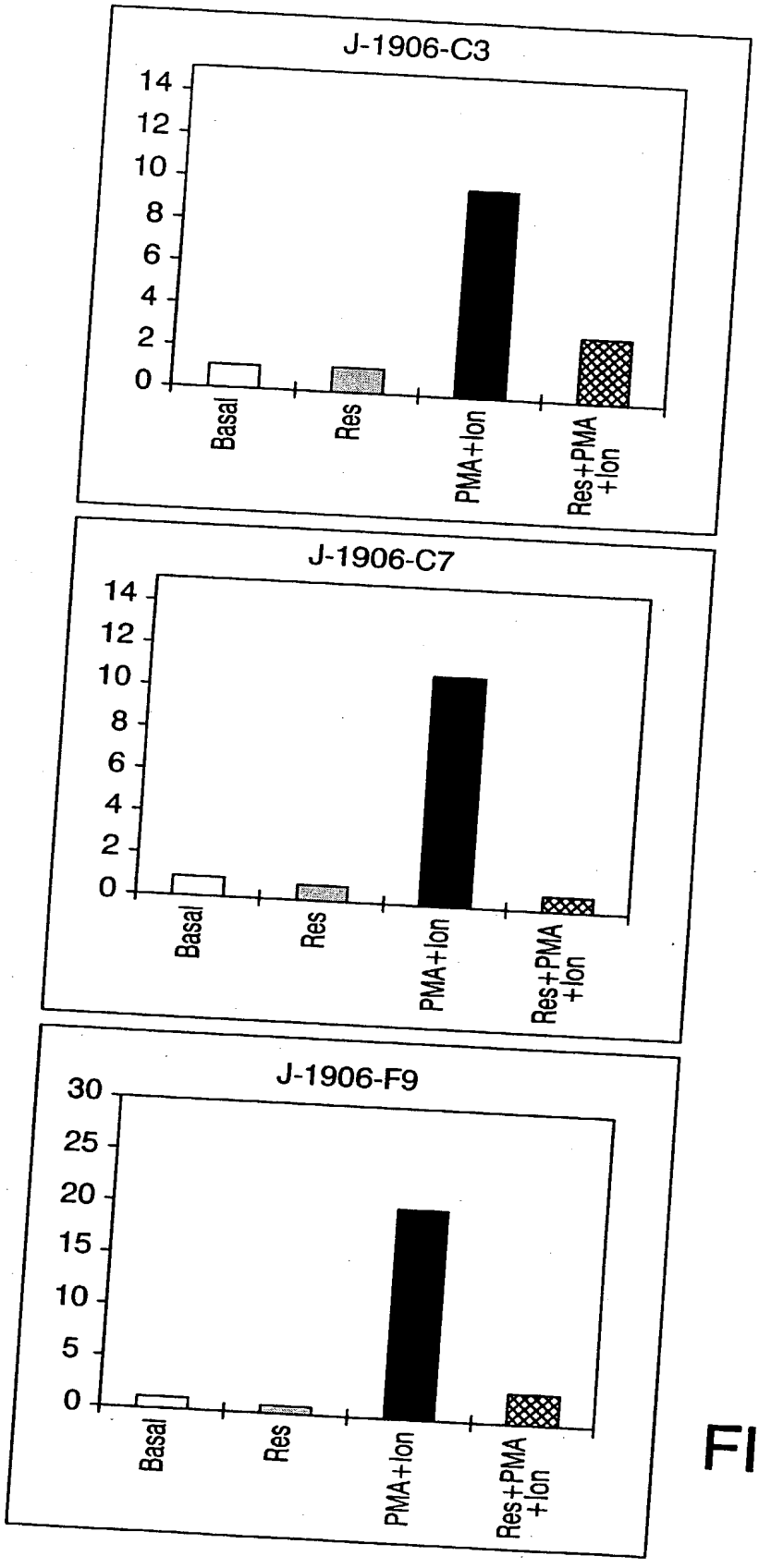
J-1906-C3
14
12
10
8
6
4
2
0
Basal
Res
PMA+Ion
Res+PMA +Ion
J-1906-C7
14
12
10
8
6
4
2
0
Basal
Res
PMA+Ion
Res+PMA +Ion
J-1906-F9
30
25
20
15
10
5
0
Basal
Res
PMA+Ion
Res+PMA +Ion

FIG. 10

DECLARATION AND POWER OF ATTORNEY

(Original, Design, National Stage of PCT, Divisional, Continuation or C-I-P Application)

As a below named inventor, I hereby declare that:
My residence, post office address and citizenship are as stated below next to my name; I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

NUCLEIC ACIDS, VECTORS, AND CELL LINES COMPRISING
A CYCLOOXYGENASE 2 (COX-2) PROMOTER AND METHODS
OF SCREENING FOR COX-2 INHIBITORS

This declaration is of the following type:

[] original
[] design
[X] national stage of PCT/ES00/00245.
[] divisional
[] continuation
[] continuation-in-part (C-I-P)

the specification of which: *(complete (a), (b), or (c))*

(a) [] is attached hereto.
(b) [X] was filed on January 10, 2002 as Application Serial No. 10/031,047 and was amended on *(if applicable).*
(c) [] was described and claimed in PCT International Application No. filed on and was amended on *(if applicable).*

Acknowledgement of Review of Papers and Duty of Candor

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of the subject matter claimed in this application in accordance with Title 37, Code of Federal Regulations § 1.56.

[ ] In compliance with this duty there is attached an information disclosure statement. 37 CFR 1.98.

Priority Claim

I hereby claim foreign priority benefits under Title 35, United States Code, § 119(a)-(d) of any foreign application(s) for patent or inventor's certificate or of any PCT International Application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT International Application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application on which priority is claimed

(complete (d) or (e))

(d) [] no such applications have been filed.
(e) [X] such applications have been filed as follows:

PRIOR FOREIGN/PCT APPLICATION(S) FILED WITHIN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO SAID APPLICATION			
COUNTRY APPLICATION NO.	DATE OF FILING (day, month, year)	DATE OF ISSUE (day, month, year)	PRIORITY CLAIMED UNDER 35 USC 119
			[] YES NO []
			[] YES NO []
			[] YES NO []
ALL FOREIGN APPLICATION[S], IF ANY, FILED MORE THAN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO SAID APPLICATION			
Spain P 9901557	12.7.99		[X] YES NO []
			[] YES NO []
			[] YES NO []

Claim for Benefit of Prior U.S. Provisional Application(s)

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below:

Provisional Application Number	Filing Date

Claim for Benefit of Earlier U.S./PCT Application(s) under 35 U.S.C. 120

(complete this part only if this is a divisional, continuation or C-I-P application)

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior application(s) in the manner provided by the first paragraph of Title 35, United States Code § 112, I acknowledge the duty to disclose information as defined in Title 37, Code of Federal Regulations, § 1.56 which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

(Application Serial No.) (Filing Date) (Status) (patented, pending, abandoned)

(Application Serial No.) (Filing Date) (Status) (patented, pending, abandoned)

Power of Attorney

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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[ ] Signature for ninth and subsequent joint inventors. Number of pages added _____.

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[ ] Signature for inventor who refuses to sign, or cannot be reached, by person authorized under 37 CFR 1.47. Number of pages added _____.